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(54) Title: DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

(57) Abstract

A method for the diagnosis of melanoma or breast cancer is provided. In particular, the method provides for the detection of nucleic acids corresponding to multiple melanoma or breast cancer specific markers using template-dependent amplification processes. In one embodiment, the markers used are tyrosinase, MUC18, p97, MAGE-3, β-HCG, MAGE-1 and GalNAc. The methods using these combinations of markers are more sensitive in the detection of tumor cells in patients as compared to single marker assays.

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DESCRIPTION

DETECTION OF MELANOMA OR BREAST METASTASES WITH A MULTIPLE MARKER ASSAY

5

BACKGROUND OF THE INVENTION

1.   Field of the Invention

The present invention relates generally to the field
15   of cancer diagnostic techniques. In particular, the
invention relates to the detection of genetic markers
indicative of melanoma or breast cancer cells. In one
example, detection of multiple markers is achieved by
polymerase chain reaction assay.

20

2.   Description of the Related Art

Cancers are one of the leading causes of disease,
being responsible for 526,000 deaths in the United States
25   each year (Boring et al., 1993). For example, breast
cancer is the most common form of malignant disease among
women in Western countries and, in the United States, is
the most common cause of death among women between 40 and
55 years of age (Forrest, 1990). The incidence of breast
30   cancer is increasing, especially in older women, but the
cause of this increase is unknown. Malignant melanoma is
another form of cancer whose incidence is increasing at a
frightening rate, at least sixfold in the United States
since 1945, and is the single most deadly of all skin
35   diseases (Fitzpatrick, 1986).

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One of the most devastating aspects of cancer is the propensity of cells from malignant neoplasms to disseminate from their primary site to distant organs and develop into metastases. Despite advances in surgical
5   treatment of primary neoplasms and aggressive therapies, most cancer patients die as a result of metastatic disease. Animal tests indicate that about 0.01% of circulating cancer cells from solid tumors establish successful metastatic colonies (Fidler, 1993).
10

Thus, the detection of occult cancer cells in circulation is important in assessing the level of tumor progression and metastasis. Because subclinical metastasis can remain dormant for many years, monitoring
15   of patients' blood for circulating tumor cells may prove advantageous in detecting tumor progression before metastasis to other organs occurs. Assessment of circulating tumor cells also would provide a rapid monitoring system to determine if a specific therapy is
20   effective.

For example, recognition of metastases in tumor-draining lymph nodes (TDLN) now has been shown critical for patient management. It is known that between 25-30
25   per cent of breast cancer patients with node negative, localized disease will relapse within five years after operative intervention (Henderson et al., 1989). Accurate axillary staging of TDLN in detection of metastases has been an important factor for selecting
30   patients for adjuvant therapy (NIH, 1992; Giuliano, et al., 1995; Giuliano, et al., 1994). Several retrospective studies on breast cancer TDLN demonstrated that analysis of multiple sections of nodes shown to be tumor negative were found to have occult metastases
35   (Bettelheim, et al., 1990; Chen et al., 1991; Neville et al., 1991). The identification of nodes with occult metastases were shown to significantly correlate to

poorer prognosis (Bettelheim et al., 1990; Neville et al., 1991).

Previous tumor diagnostic techniques have focused on the detection of tumor associated antigens or on molecules released by tumor cells (Smart, 1990; Moertel et al., 1993; Stamey et al., 1989). At best, these assays only detect tumors with no indication of metastatic potential or tumor progression. In addition, such assays measure a single antigen whose release is often proportional to the size of the tumor and they cannot account for heterogeneity of individual markers in tumor lesions, both within individual patients or among patient groups.

The recent development of the PCR assay (Mullis and Faloona, 1987; Erlich, 1989) for detection of occult metastatic tumor cells in blood using specific markers has provided a new approach to assess tumor progression (Smith et al., 1991; Naito et al., 1991). In one study, circulating melanoma cells in blood were detected by PCR analysis using the tyrosinase gene marker (Smith et al., 1991). Seven melanoma patients with metastatic disease were analyzed, but only four were positive. Other studies using PCR have been used to detect circulating tumor cells in melanoma, as well as in breast, prostate and neuroblastoma cancer patients (Smith et al., 1991; Datta et al., 1994; Moreno et al., 1992; Naito et al., 1991). These studies, employing a single marker, were limited by their ability to discriminate cancer cells from normal cells also carrying the marker, thus reducing specificity and reliability. In addition, tumor heterogeneity has caused sensitivity problems where a single, specific marker has been employed.

As indicated above, tumors are notoriously heterogeneous, particularly in advanced stages of tumor

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progression (Morton *et al.*, 1993; Fidler and Hart, 1982;
Nowell, 1982; Elder *et al.*, 1989; Bystryn *et al.*, 1985).
Although tumor cells within a primary tumor or metastasis
all may express the same marker gene, the level of
5   specific mRNA expression can vary considerably (Elder
et al., 1989). It is, therefore, necessary to develop a
detection system that can cope with such heterogeneous
targets.

10   Thus, despite the identification of melanoma and
breast cancer markers, these markers cannot individually
detect tumor cells in a highly specific and sensitive
manner. This is due to the wide phenotypic diversity
found in tumor cells at any one time and during disease
15   differentiation. There remains a need to develop a more
sophisticated approach, that can accommodate such a
biological heterogeneous situation in order to
sensitively and specifically detect metastasis and
diagnosis disease stage.
20

3.   **Summary of the Invention**

The present invention seeks to overcome these and
other drawbacks inherent in the prior art by providing
25   sensitive and accurate methods for the detection of
melanoma or breast cancer cells in a biological sample.
The methods provide for the detection of melanoma or
breast cancer cells in a biological sample by amplifying
at least two nucleic acids from the sample, the nucleic
30   acids being markers for melanoma or breast cancer cells.

The present invention comprises the following steps.
A nucleic acid is extracted from a biological sample.
The nucleic acid is contacted with a first primer pair
35   that hybridizes to a first melanoma or breast cancer
marker nucleic acid. The primers are extended by
polymerase to produce an amplification product. This

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process is repeated a sufficient number of times to
permit detection of the amplification product. Finally,
all steps are repeated with the same biological sample
and a second primer pair that hybridizes to another
5   melanoma or breast cancer marker nucleic acid.

       In preferred embodiments, the method may further
comprise preparing at least two pairs of primers
complementary to regions of melanoma or breast cancer
10   marker nucleic acids. In another embodiment, the method
may further comprise of preparing primer pairs for at
least three, four, five, six or even seven melanoma or
breast cancer markers.

15   In preferred embodiments of the invention, the
markers amplified and detected are selected from the
group comprising tyrosinase, MAGE-3, MUC18, p97, MAGE-1,
GalNAc and β-HCG. The preferred method of amplification
is by reverse transcription and polymerase chain reaction
20   (PCR). In one embodiment of the invention the PCR
further comprises nested PCR.

       In one embodiment, the nucleic acid is RNA.
Preferably, the RNA extracted from a biological sample is
25   total cellular RNA. In a preferred embodiment, the total
cellular RNA is converted to DNA prior to amplification.

       In certain embodiments of the invention, the
biological sample is a body tissue or body fluid. In
30   preferred embodiments, the body tissue is bone marrow
aspirate, bone marrow biopsy, lymph node aspirate, lymph
node biopsy, spleen tissue, fine needle aspirate, skin
biopsy or organ tissue biopsy. Other embodiments
include samples where the body fluid is peripheral blood,
35   lymph fluid, ascites, serous fluid, pleural effusion,
sputum, cerebrospinal fluid, lacrimal fluid, stool or

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urine. In a preferred embodiment, the biological sample
is of human origin.

In preferred embodiments of the invention, the
5   method includes separation of the amplification product
by gel electrophoresis. In other embodiments, the method
of separation is by chromatographic techniques. In a
preferred embodiment of the invention, hybridization with
a labeled probe permits identification of the
10   amplification product following separation.

In further embodiments, the present invention
encompasses a kit for use in detecting melanoma or breast
cancer cells in a biological sample comprising, pairs of
15   primers for amplifying nucleic acids corresponding to the
marker genes, and containers for each of these primers.
In preferred embodiments, the kit further comprises
enzymes and reagents for the preparation of cDNA's and
amplification thereof. In yet more preferred
20   embodiments, the kit further comprises enzymes and
reagents for radiochemical or chromophoric labeling of
nucleic acids.

4.   **Detailed Description of the Preferred Embodiments**
25
The present invention pertains to a sensitive,
multimarker assay to detect occult melanoma or breast
cancer cells in the blood of patients with or without
clinical evidence of disease. This assay is designed to
30   overcome limitations in existing technologies with
respect to both sensitivity and specificity.

In its most general form, the instant invention
comprises a method for identification of melanoma or
35   breast cancer cells in a biological sample by amplifying
and detecting nucleic acids corresponding to melanoma or
breast cancer cell markers. The biological sample can be

any tissue or fluid in which melanoma or breast cancer
cells might be present. Preferred embodiments include
bone marrow aspirate, bone marrow biopsy, lymph node
aspirate, lymph node biopsy, spleen tissue, fine needle

5   aspirate, skin biopsy or organ tissue biopsy. Other
embodiments include samples where the body fluid is
peripheral blood, lymph fluid, ascites, serous fluid,
pleural effusion, sputum, cerebrospinal fluid, lacrimal
fluid, stool or urine.

10

Nucleic acid used as template for amplification is
isolated from cells contained in the biological sample
according to standard methodologies. (Sambrook et al.,
1989) The nucleic acid may be genomic DNA or

15   fractionated or whole cell RNA. Where RNA is used, it
may be desired to convert the RNA to a complementary
cDNA. In a preferred embodiment, the RNA is whole cell
RNA and is used directly as the template for
amplification.

20

Pairs of primers that selectively hybridize to genes
corresponding to specific markers are contacted with the
isolated nucleic acid under conditions that permit
selective hybridization. Once hybridized, the nucleic

25   acid:primer complex is contacted with one or more enzymes
that facilitate template-dependent nucleic acid
synthesis. Multiple rounds of amplification, also
referred to as "cycles," are conducted until a sufficient
amount of amplification product is produced.

30

Next, the amplification product is detected. In
certain applications, the detection may be performed by
visual means. Alternatively, the detection may involve
indirect identification of the product via

35   chemiluminescence, radioactive scintigraphy of
incorporated radiolabel or fluorescent label or even via

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a system using electrical or thermal impulse signals
(Affymax technology, Bellus, 1994).

The foregoing process is conducted at least twice on
5   a given sample using at least two different primer pairs
specific for two different specific markers. Following
detection, one may compare the results seen in a given
patient with a statistically significant reference group
of normal patients and melanoma or breast cancer
10   patients. In this way, it is possible to correlate the
number and kind of markers with various clinical states.

(i) *Melanoma-Specific or Breast Cancer-Specific
Markers*

15

While the present invention exemplifies several
markers, any marker that is correlated with the presence
or absence of melanomas or breast cancer may be used. A
marker, as used herein, is any proteinaceous molecule (or
20   corresponding gene) whose production or lack of
production is characteristic of a melanoma or breast
cancer cell. Depending on the particular set of markers
employed in a given analysis, the statistical analysis
will vary. For example, where a particular combination
25   of markers is highly specific for melanomas or breast
cancer, the statistical significance of a positive result
will be high. It may be, however, that such specificity
is achieved at the cost of sensitivity, *i.e.*, a negative
result may occur even in the presence of melanoma or
30   breast cancer. By the same token, a different
combination may be very sensitive, *i.e.*, few false
negatives, but has a lower specificity.

As new markers are identified, different
35   combinations may be developed that show optimal function
with different ethnic groups or sex, different geographic
distributions, different stages of disease, different

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degrees of specificity or different degrees of
sensitivity. Marker combinations may also be developed,
which are particularly sensitive to the effect of
therapeutic regimens on disease progression. Patients
5   may be monitored after surgery, hyperthermia,
immunotherapy, cytokine therapy, gene therapy,
radiotherapy or chemotherapy, to determine if a specific
therapy is effective.

10   One particularly useful combination of markers is
tyrosinase and p97. Human tyrosinase is an essential
enzyme which regulates the production of melanin
(Nordlund et al., 1989; Hoon et al., 1993), a group of
brown or black pigments in the skin and eyes of humans.
15   More specifically, tyrosinase catalyzes the conversion of
tyrosine to Dopa and of Dopa to dopaquinone. p97, also
known as melanotransferrin, is a cell surface
sialoglycoprotein that bears some sequence homology to
transferrin (Brown et al., 1981; Rose et al., 1986).
20   Like transferrin, p97 binds iron, thereby being
implicated in iron metabolism.

There are many other markers that may be used in
combination with these, and other, markers. For example,
25   β-human chorionic gonadotropin (β-HCG). β-HCG is
produced by trophoblastic cells of placenta of pregnant
woman and is essential for maintenance of pregnancy at
the early stages (Pierce et al., 1981; Talmadge et al.,
1984). β-HCG is known to be produced by trophoblastic or
30   germ cell origin tumors, such as choriocarcinoma or
testicular carcinoma cells (Madersbacher et al., 1994;
Cole et al., 1983). Also ectopic expression of β-HCG has
been detected by a number of different immunoassays in
various tumors of non-gonadal such as breast, lung,
35   gastric, colon, and pancreas, etc. (McManus et al., 1976;
Yoshimura et al., 1994; Yamaguchi et al., 1989; Marcillac
et al., 1992; Alfthan et al., 1992). Although the

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function of β-HCG production in these tumors is still
unknown, the atavistic expression of β-HCG by cancer
cells and not by normal cells of non-gonadal origin
suggests it may be a potentially good marker in the
5   detection of melanoma and breast cancer (Tormey et al.,
1977; Tormey et al., 1975).

Another exemplary example of a marker is
glycosyltransferase β-1, 4-N-acetylgalacto-
10   saminyltransferase (GalNAc). GalNAc catalyzes the
transfer of N-acetylgalactosamine by β1,4 linkage onto
both gangliosides GM3 and GD3 to generate GM2 and GD2,
respectively (Nagata, et al., 1992; Furukawa et al.,
1993). It also catalyzes the transfer of N-
15   acetylgalactosamine to other carbohydrate molecules such
as mucins. Gangliosides are glycosphingolipids
containing sialic acids which play an important role in
cell differentiation, adhesion and malignant
transformation. In melanoma, gangliosides GM2 and GD2
20   expression, are often enhanced to very high levels and
associated with tumor progression including metastatic
tumors (Hoon et al., 1989; Ando et al., 1987; Carubia
et al., 1984; Tsuchida et al., 1987a). Gangliosides are
also highly expressed in breast cancer cells. The
25   gangliosides GM2 and GD2 are immunogenic in humans and
can be used as a target for specific immunotherapy such
as human monoclonal antibodies or cancer vaccines
(Tsuchida et al., 1987b; Irie, 1985).

30   GalNAc mRNA may be used as a marker of GM2 and GD2
expression and consequently a marker of either melanoma
or breast cancer cells. GalNAc is generally not
expressed in normal lymphocytes, epithelial cells,
melanocytes, connective tisue or lymph node cells. If
35   detected, it is in very low levels.

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Other markers contemplated by the present invention
include cytolytic T lymphocyte (CTL) targets. MAGE-3 is
a marker identified in melanoma cells and breast
carcinoma. MAGE-3 is expressed in many melanomas as well
5   as other tumors and is a (CTL) target (Gaugler et al.,
1994). MAGE-1 and MAGE-2 are other members of the MAGE
gene family. MAGE-1 gene sequence shows 73% identity
with MAGE-3 and expresses an antigen also recognized by
CTL (Gaugler et al., 1994). MART-1 is another potential
10 CTL target (Robbins et al., 1994) and·may also be
included in the present invention.

MUC18 is another marker that is useful in the
identification of melanoma cells (Lehmann et al., 1989;
15 Lehmann et al., 1987). MUC18 is a cell surface
glycoprotein that is a member of the immunoglobulin
superfamily and possesses sequence homology to neural
cell adhesion molecules (NCAM). Other mucin family
members include MUC1, MUC2, MUC3 and MUC4. These were
20 found to be expressed at a high level in certain tumor
cell lines (Hollingsworth et al., 1994) and may also be
used as markers in the present invention.

Other members of the immunoglobulin superfamily of
25 adhesions molecules associated with the development of
melanoma metastasis (Denton et al., 1992) may be utilized
in the present invention. Preferred examples include
intercellular adhesion molecule-1 (ICAM-1), NCAM, VCAM-1,
and ELAM. Another preferred embodiment of the invention,
30 includes cell adhesion molecules associated with other
metastatic diseases, such as carcinoembryonic antigen
(CEA) and DCC (deleted in colorectal cancer) (Johnson,
1991).

35 Other breast or skin cancer associated proteins and
their corresponding nucleic acids may also be utilized in
the present invention. Preferred examples include

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melanoma antigen gp75 (Vijayasardahi *et al.*, 1990), human
cytokeratin 8 (HKer 8) (Pittman *et al.*, 1993), high
molecular weight melanoma antigen (Natali *et al.*, 1987)
and Keratin 19 (K19) (Datta *et al.*, 1994). This list is
5   not intended to be exhaustive, but merely exemplary, for
the type and number of potential markers which may be
used in the present invention.

Other proteins and their corresponding nucleic acids
10   related to the melanin synthesis pathway may be used as
markers, such as tyrosinase related protein 1 and 2 and
members of the pMel 17 gene family (Kwon *et al.*, 1993).

Preferred embodiments of the invention involve many
15   different combinations of markers for the detection of
melanoma breast cancer cells. Any marker that is
indicative of neoplasia in breast cells or melanocytes
may be included in this invention. However, preferred
embodiments include combinations of tyrosinase, MAGE-3,
20   MUC18, p97, β-HCG, GalNAc and MAGE-1. Table 1, as
disclosed herein, represent partially useful combinations
of markers which may be employed for the detection of
melanoma or breast cancer cells.

Table 1. Preferred Multiple Marker Combinations
Table 1A. Combinations of Six or Seven Multiple Markers.

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	+	+
+	+	+	+	+	+	-
+	+	+	+	+	-	+
+	+	+	+	-	+	+
+	+	+	-	+	+	+
+	+	-	+	+	+	+
+	-	+	+	+	+	+
-	+	+	+	+	+	+

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Table 1B. Combinations of Five Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	+	−	−
+	+	+	+	−	−	+
+	+	+	−	−	+	+
+	+	−	−	+	+	+
+	−	−	+	+	+	+
−	−	+	+	+	+	+
−	+	+	+	+	+	−
+	+	+	+	−	+	−
+	+	+	−	+	−	+
+	+	−	+	−	+	+
+	−	+	−	+	+	+
−	+	−	+	+	+	+
+	−	+	+	+	+	−
−	+	+	+	+	−	+
+	+	+	−	+	+	−
+	+	−	+	+	−	+
+	−	+	+	−	+	+
−	+	+	−	+	+	+
+	+	−	+	+	+	−
+	−	+	+	+	−	+
−	+	+	+	−	+	+

Table 1C. Combinations of Four Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
+	+	+	+	-	-	-
+	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	+	+
-	-	-	+	+	+	+
-	-	+	+	+	+	-
-	+	+	+	+	-	-
+	+	+	-	-	+	-
+	+	-	-	+	-	+
+	-	-	+	-	+	+
-	-	+	-	+	+	+
-	+	-	+	+	+	-
+	-	+	+	+	-	-
-	+	+	+	-	-	+
+	+	+	-	+	-	-
+	+	-	+	-	-	+
+	-	+	-	-	+	+
-	+	-	-	+	+	+
+	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	+	-
+	+	-	-	+	+	-
+	-	-	+	+	-	+
-	-	+	+	-	+	+
-	+	+	-	+	+	-
+	+	-	+	+	-	-
+	-	+	+	-	-	+
-	-	+	-	-	+	+

Table 1D. Combinations of Three Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	+	+	+
-	-	-	+	+	+	-
-	-	+	+	+	-	-
-	+	+	+	-	-	-
+	+	+	-	-	-	-
+	+	-	-	-	-	+
+	-	-	-	-	+	+
-	-	-	+	+	-	+
-	-	+	+	-	+	-
-	+	+	-	+	-	-
+	+	-	+	-	-	-
+	-	+	-	-	-	+
-	+	-	-	-	+	+
+	-	-	-	+	+	-
-	-	-	+	-	+	+
-	-	+	-	+	+	-
-	+	-	+	+	-	-
+	-	+	+	-	-	-
-	+	+	-	-	-	+
+	+	-	-	-	+	+
+	-	-	-	+	-	+
-	-	+	+	-	-	+
-	+	+	-	-	+	-
+	+	-	-	+	-	-
+	-	-	+	-	-	+
-	-	+	-	-	+	+
-	+	-	-	+	+	-
+	+	-	+	+	-	-

Table 1E. Combinations of Two Multiple Markers

Tyrosinase	p97	MUC18	MAGE3	β-HCG	GluNAc	MAGE1
-	-	-	-	-	+	+
-	-	-	-	+	+	-
-	-	-	+	+	-	-
-	-	+	+	-	-	-
-	+	+	-	-	-	-
+	+	-	-	-	-	-
+	-	-	-	-	-	+
-	-	-	-	+	-	+
-	-	-	+	-	+	-
-	-	+	-	+	-	-
-	+	-	+	-	-	-
+	-	+	-	-	-	-
-	+	-	-	-	-	+
+	-	-	-	-	+	-
-	-	-	+	-	-	+
-	-	+	-	-	+	-
-	+	-	-	+	-	-
+	-	-	+	-	-	-
-	-	+	-	-	-	+
-	+	-	-	-	+	-
+	-	-	-	+	-	-

+ markers included in the combination; - markers not
included.

(ii) Primers

The term primer, as defined herein, is meant to
encompass any nucleic acid that is capable of priming the
synthesis of a nascent nucleic acid in a template-
dependent process. Typically, primers are
oligonucleotides from ten to twenty base pairs in length,
but longer sequences can be employed. Primers may be

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provided in double-stranded or single-stranded form,
although the single-stranded form is preferred.

In most cases, it will be preferable to synthesize
5   desired oligonucleotides. Suitable primers can be
synthesized using commercial synthesizers, such as those
supplied by Applied Biosystems (Foster City, CA) using
methods well known to those of ordinary skill in the art.
Where double-stranded primers are desired, synthesis of
10 complementary primers is performed separately and the
primers mixed under conditions permitting their
hybridization.

Selection of primers is based on a variety of
15 different factors, depending on the method of
amplification and the specific marker involved. For
example, the choice of primer will determine the
specificity of the amplification reaction. The primer
needs to be sufficiently long to specifically hybridize
20 to the marker nucleic acid and allow synthesis of
amplification products in the presence of the
polymerization agent and under appropriate temperature
conditions. Shorter primer molecules generally require
cooler temperatures to form sufficiently stable hybrid
25 complexes with the marker nucleic acid and may be more
susceptible to non-specific hybridization and
amplification.

Primer sequences do not need to correspond exactly
30 to the specific marker sequence. Non-complementary
nucleotide fragments may be attached to the 5' end of the
primer with the remainder of the primer sequence being
complementary to the template. Alternatively, non-
complementary bases can be interspersed into the primer,
35 provided that the primer sequence has sufficient
complementarily, in particular at the 3' end, with the

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template for annealing to occur and allow synthesis of a
complementary DNA strand.

5   In preferred embodiments, primers may be designed to
hybridize to specific regions of the marker nucleic acid
sequence. For example, GC rich regions are favored as
they form stronger hybridization complexes than AT rich
regions. In another example, primers are designed,
solely, to hybridize to a pair of exon sequences, with at
10   least one intron in between. This allows for the
activity of a marker gene to be detected as opposed to
its presence by minimizing background amplification of
the genomic sequences and readily distinguishes the
target amplification by size.
15

Primers also may be designed to amplify a particular
segment of marker nucleic acid that encodes restriction
sites. A restriction site in the final amplification
product would enable digestion at that particular site by
20   the relevant restriction enzyme to produce two products
of a specific size. Any restriction enzyme may be
utilized in this aspect. This added refinement to the
amplification process may be necessary when amplifying a
marker nucleic acid sequence with close sequence
25   similarity to other nucleic acids. Alternatively, it may
be used as an added confirmation of the specificity of
the amplification product.

(iii)   Template Dependent Amplification Methods
30

A number of template dependent processes are
available to amplify the marker sequences present in a
given template sample. One of the best known
amplification methods is the polymerase chain reaction
35   (referred to as PCR) which is described in detail in U.S.
Patent Nos. 4,683,195, 4,683,202 and 4,800,159, and in
Innis et al., 1990, each of which is incorporated herein

- 20 -

by reference in its entirety. Briefly, in PCR, two
primer sequences are prepared which are complementary to
regions on opposite complementary strands of the marker
sequence. An excess of deoxynucleoside triphosphates are
5   added to a reaction mixture along with a DNA polymerase,
e.g., *Taq* polymerase. If the marker sequence is present
in a sample, the primers will bind to the marker and the
polymerase will cause the primers to be extended along
the marker sequence by adding on nucleotides. By raising
10   and lowering the temperature of the reaction mixture, the
extended primers will dissociate from the marker to form
reaction products, excess primers will bind to the marker
and to the reaction products and the process is repeated.
Preferably a reverse transcriptase PCR amplification
15   procedure may be performed in order to quantify the
amount of mRNA amplified. Methods of reverse
transcribing RNA into cDNA are well known and described
in Sambrook *et al.*, 1989. Alternatively, preferred
methods for reverse transcription utilize thermostable
20   DNA polymerases. These methods are described in WO
90/07641 filed December 21, 1990. Polymerase chain
reaction methodologies are well known in the art.

Another method for amplification is the ligase chain
25   reaction ("LCR"), disclosed in EPO No. 320 308,
incorporated herein by reference in its entirety. In
LCR, two complementary probe pairs are prepared, and in
the presence of the marker sequence, each pair will bind
to opposite complementary strands of the marker such that
30   they abut. In the presence of a ligase, the two probe
pairs will link to form a single unit. By temperature
cycling, as in PCR, bound ligated units dissociate from
the marker and then serve as "target sequences" for
ligation of excess probe pairs. U.S. Patent
35   No. 4,883,750 describes a method similar to LCR for
binding probe pairs to a marker sequence.

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Qbeta Replicase, described in PCT Application No.
PCT/US87/00880, may also be used as still another
amplification method in the present invention. In this
method, a replicative sequence of RNA which has a region
5   complementary to that of a marker is added to a sample in
the presence of an RNA polymerase. The polymerase will
copy the replicative sequence which can then be detected.

An isothermal amplification method, in which
10   restriction endonucleases and ligases are used to achieve
the amplification of marker molecules that contain
nucleotide 5'-[alpha-thio]-triphosphates in one strand of
a restriction site may also be useful in the
amplification of nucleic acids in the present invention.
15   Walker et al., 1992, incorporated herein by reference in
its entirety.

Strand Displacement Amplification (SDA) is another
method of carrying out isothermal amplification of
20   nucleic acids which involves multiple rounds of strand
displacement and synthesis, i.e., nick translation. A
similar method, called Repair Chain Reaction (RCR)
involves annealing several probes throughout a region
targeted for amplification, followed by a repair reaction
25   in which only two of the four bases are present. The
other two bases can be added as biotinylated derivatives
for easy detection. A similar approach is used in SDA.
Marker specific sequences can also be detected using a
cyclic probe reaction (CPR). In CPR, a probe having a 3'
30   and 5' sequences of non-specific DNA and middle sequence
of specific RNA is hybridized to DNA which is present in
a sample. Upon hybridization, the reaction is treated
with RNaseH, and the products of the probe identified as
distinctive products which are released after digestion.
35   The original template is annealed to another cycling
probe and the reaction is repeated.

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Still another amplification methods described in
British Patent Application No. 2,202,328, and in PCT
Application No. PCT/US89/01025, each of which is
incorporated herein by reference in its entirety, may be
5   used in accordance with the present invention. In the
former application, "modified" primers are used in a PCR
like, template and enzyme dependent synthesis. The
primers may be modified by labelling with a capture
moiety (e.g., biotin) and/or a detector moiety (e.g.,
10   enzyme). In the latter application, an excess of labeled
probes are added to a sample. In the presence of the
marker sequence, the probe binds and is cleaved
catalytically. After cleavage, the marker sequence is
released intact to be bound by excess probe. Cleavage of
15   the labeled probe signals the presence of the marker
sequence.

Other nucleic acid amplification procedures include
transcription-based amplification systems (TAS),
20   including nucleic acid sequence based amplification
(NASBA) and 3SR. Kwoh et al., 1989; Gingeras et al., PCT
Application WO 88/10315, incorporated herein by reference
in their entirety. In NASBA, the nucleic acids can be
prepared for amplification by standard phenol/chloroform
25   extraction, heat denaturation of a clinical sample,
treatment with lysis buffer and minispin columns for
isolation of DNA and RNA or guanidinium chloride
extraction of RNA. These amplification techniques
involve annealing a primer which has marker specific
30   sequences. Following polymerization, DNA/RNA hybrids are
digested with RNase H while double stranded DNA molecules
are heat denatured again. In either case the single
stranded DNA is made fully double stranded by addition of
second marker specific primer, followed by
35   polymerization. The double-stranded DNA molecules are
then multiply transcribed by a polymerase such as T7 or
SP6. In an isothermal cyclic reaction, the RNAs are

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reverse transcribed into double-stranded DNA, and
transcribed once against with a polymerase such as T7 or
SP6. The resulting products, whether truncated or
complete, indicate marker specific sequences.

5

        Davey et al., EPO No. 329 822 (incorporated herein
by reference in its entirety) disclose a nucleic acid
amplification process involving cyclically synthesizing
single-stranded RNA ("ssRNA"), ssDNA, and double-stranded

10   DNA (dsDNA), which may be used in accordance with the
present invention. The ssRNA is a first template for a
first primer oligonucleotide, which is elongated by
reverse transcriptase (RNA-dependent DNA polymerase).
The RNA is then removed from the resulting DNA:RNA duplex

15   by the action of ribonuclease H (RNase H, an RNase
specific for RNA in duplex with either DNA or RNA). The
resultant ssDNA is a second template for a second primer,
which also includes the sequences of an RNA polymerase
promoter (exemplified by T7 RNA polymerase) 5' to its

20   homology to the template. This primer is then extended
by DNA polymerase (exemplified by the large "Klenow"
fragment of E. coli DNA polymerase I), resulting as a
double-stranded DNA ("dsDNA") molecule, having a sequence
identical to that of the original RNA between the primers

25   and having additionally, at one end, a promoter sequence.
This promoter sequence can be used by the appropriate RNA
polymerase to make many RNA copies of the DNA. These
copies can then re-enter the cycle leading to very swift
amplification. With proper choice of enzymes, this

30   amplification can be done isothermally without addition
of enzymes at each cycle. Because of the cyclical nature
of this process, the starting sequence can be chosen to
be in the form of either DNA or RNA.

35   Miller et al., PCT Application WO 89/06700
(incorporated herein by reference in its entirety)
disclose a nucleic acid sequence amplification scheme

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based on the hybridization of a promoter/primer sequence
to a marker single-stranded DNA ("ssDNA") followed by
transcription of many RNA copies of the sequence. This
scheme is not cyclic, *i.e.*, new templates are not
5   produced from the resultant RNA transcripts. Other
amplification methods include "race" and "one-sided PCR."
Frohman, M.A., 1990 and Ohara *et al.*, 1989, each
incorporated herein by reference in their entirety.

10   Methods based on ligation of two (or more)
oligonucleotides in the presence of nucleic acid having
the sequence of the resulting "di-oligonucleotide",
thereby amplifying the di-oligonucleotide, may also be
used in the amplification step of the present invention.
15   Wu *et al.*, 1989, incorporated herein by reference in its
entirety.

       (iv)   Separation Methods

20   Following amplification, it may be desirable to
separate the amplification product from the template and
the excess primer for the purpose of determining whether
specific amplification occurred. In a preferred
embodiment, amplification products are separated by
25   agarose, agarose-acrylamide or polyacrylamide gel
electrophoresis using standard methods. See Sambrook *et
al.*, 1989. In a preferred embodiment, the gel is a 2%
agarose gel.

30   Alternatively, chromatographic techniques may be
employed to effect separation. There are many kinds of
chromatography which may be used in the present
invention: adsorption, partition, ion-exchange and
molecular sieve, and many specialized techniques for
35   using them including column, paper, thin-layer and gas
chromatography (Freifelder, 1982).

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(v)   Identification Methods

     Amplification products must be visualized in order
to confirm amplification of the marker sequences. One
5   typical visualization method involves staining of a gel
with ethidium bromide and visualization under UV light.
Alternatively, if the amplification products are
integrally labeled with radio- or fluorometrically-
labeled nucleotides, the amplification products can then
10   be exposed to x-ray film or visualized under the
appropriate stimulating spectra, following separation.

     Alternatively, separation may be unnecessary. These
methods may be collectively termed Sequencing By
15   Hybridization or SBH (Cantor et al., 1992; Drmanac &
Crkvenjakov, U.S. Patent No. 5,202,231). Development of
certain of these methods has given rise to new solid
support type sequencing tools known as sequencing chips.
The utility of SBH in general is evidenced by the fact
20   that U.S. Patents have been granted on this technology.

     SBH can be conducted in two basic ways, often
referred to as Format 1 and Format 2 (Cantor et al.,
1992). In Format 1, oligonucleotides of unknown
25   sequence, generally of about 100-1000 nucleotides in
length, are arrayed on a solid support or filter so that
the unknown samples themselves are immobilized (Strezoska
et al., 1991; Drmanac & Crkvenjakov, U.S. Patent No.
5,202,231). Replicas of the array are then interrogated
30   by hybridization with sets of labeled probes of about 6
to 8 residues in length.

     In Format 2, a sequencing chip is formed from an
array of oligonucleotides with known sequences of about 6
35   to 8 residues in length (Southern, WO 89/10977; Khrapko
et al., 1991; Southern et al., 1992). The nucleic acids
of unknown sequence are then labeled and allowed to

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hybridize to the immobilized oligos. In another
embodiment, hybridization may be detected by electrical
or thermal impulse signals (Affymax Technology, Bellus,
1994).

5

In a preferred method, however, visualization is
achieved indirectly. Following separation of
amplification products, a labeled, nucleic acid probe is
brought into contact with the amplified marker sequence.
10   The probe preferably is conjugated to a chromophore but
may be radiolabeled. In another embodiment, the probe is
conjugated to a binding partner, such as an antibody or
biotin, where the other member of the binding pair
carries a detectable moiety.

15

In a particularly preferred embodiment, detection is
by Southern blotting and hybridization with a labeled
probe. The techniques involved in Southern blotting are
well known to those of skill in the art and can be found
20   in many standard books on molecular protocols. See
Sambrook et al., 1989. Briefly, amplification products
are separated by gel electrophoresis. The gel is then
contacted with a membrane, such as nitrocellulose,
permitting transfer of the nucleic acid and non-covalent
25   binding. Subsequently, the membrane is incubated with a
chromophore conjugated probe that is capable of
hybridizing with a target amplification product.
Detection is by exposure of the membrane to x-ray film or
ion-emitting detection devices.

30

One example of the foregoing is described in U.S.
Patent No. 5,279,721, incorporated by reference herein,
which discloses an apparatus and method for the automated
electrophoresis and transfer of nucleic acids. The
35   apparatus permits electrophoresis and blotting without
external manipulation of the gel and is ideally suited to

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carrying out methods according to the present invention.

(vi) Clinical Stages of Malignant Melanoma

5   Cancers are staged according to a well-defined,
elaborate progressive scale, developed by the American
Joint Committee on Cancer.

        Malignant melanomas can arise in any skin area that
10   contains melanocytes, but body moles, also called
pigmented nevi, are particularly vulnerable. Although
some moles, especially those on the face and torso,
originate in pigment cells, they sometimes contain little
pigment and are light in color. All moles are initially
15   benign tumors of varying shape, but it is significant to
note that about 20 to 30 percent of all melanomas begin
in the pigment cells of moles.

        Caught early, melanoma is very often curable. On
20   the other hand, melanomas that are not detected until
they have invaded even a few millimeters of the deeper
layers of skin have a much poorer prognosis. The five-
year survival rate varies considerably depending on stage
level. For Stage I and Stage II melanoma, the five-year
25   survival rate is over 80%. However, for Stage IV the
survival rate is less that 20% (AJCC, .

        A simplified summary of the scale, developed by the
American Joint Committee for the Staging of melanoma is
30   presented in Table 2.

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Table 2: Staging of Melanoma

Stage I: Primary site, small tumor Negative lymph nodes No detectable metastases
Stage II: Invasion beyound primary site Lymph nodes negative may have one positive No detectable distant metastases
Stage III: Tumors at regional skin or subcutaneous sites, primarily located to lymph nodes.
Stage IV: Tumor of any size Lymph nodes either positive or negative Distant metastases to multiple sites

Metastasis to a distal organ may or may not result in secondary metastasis to other organs. Since subclinical metastasis can remain dormant for many years, monitoring of a patient's blood for circulating tumor cells may be helpful in detecting tumor progression before clinically evident metastases to other organs are detected.

(vii) Clinical Stages of Breast Cancer

Many factors appear to influence the chances of surviving breast cancer. Early detection and treatment are the most important. The overall five-year survival rate is about 75 percent for white women and about 63 percent for black women. This rises to nearly 90 percent for women with Stage I or II cancer that is treated while the cancer is confined to the breast (Scanlon and Strax, 1986).

A simplified summary of the scale, developed by the American Joint Committee for the Staging of Breast Cancer in 1982, is presented in Table 3.

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Table 3: Staging of Breast Cancers

Stage I: Small tumor (less than 2 cm or .78 inches)
     Negative lymph nodes
     No detectable metastases

5

Stage II: Tumor greater than 2 cm but less than 5 cm
     Lymph nodes negative
          or
     Tumor less than 5 cm across
     Lymph nodes positive
     ·No detectable distant metastases

10

Stage III: Large tumor (greater than 5 cm)
          · or
     Tumor of any size with invasion of skin or chest
     wall or "grave signs"
          or
     Associated with positive lymph nodes in the
     collarbone area
          but
     No detectable distant metastases

15

20

Stage IV: Tumor of any size
     Lymph nodes either positive or negative
     Distant metastases

(viii)   Kit Components

25

All the basic essential materials and reagents required for detecting melanoma or breast cancer cells in a biological sample, may be assembled together in a kit. This will generally comprise of the preselected primers

30   for two, or more, particular specific markers. Also included may be enzymes suitable for amplifying nucleic acids including various polymerases (RT, Taq, etc.), deoxynucleotides and buffers to provide the necessary reaction mixture for amplification.

35

Such kits will generally comprise, in suitable means, distinct containers for each individual reagent and enzyme as well as for each marker primer pair. Preferred pairs of primers for amplifying nucleic acids

40   correspond to the genes for tyrosinase, MAGE-3, MUC18, p97, MAGE-1, GalNAc and β-HCG.

- 30 -

Kits of the present invention, also will typically include a means for containing the reagents in close confinement for commercial sale such as, *e.g.*, injection or blow-molded plastic containers into which the desired·
5   reagent are retained. Other containers suitable for conducting certain steps of the disclosed methods also may be provided.

5.   **Examples**
10

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow
15   represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many
20   changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

25   **EXAMPLE I**
                 Detection of Multiple Marker RNA Expression
                            in Melanoma Cells

A.   **MATERIALS AND METHODS**
30

(i)   *Melanoma cell lines*

Melanoma cell lines M10, M12, M24, M101, Mke, Mst, Mmu, Mka, and Mkn were established and characterized at
35   the John Wayne Cancer Institute (JWCI). Cells were grown in RPMI 1640 plus 10% fetal calf serum (heat-inactivated) (Gemini, Calabasas, CA) plus penicillin and streptomycin

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(GIBCO, Long Island, NY) in T75 cm^2 flasks. Adherent
cell lines were routinely passaged by trypsinization
every 3-4 days (Hoon *et al.*, 1993). For PCR studies,
cell lines were used when 75-85% confluent.

5

   (ii) Blood preparation and RNA extraction

      Peripheral blood lymphocytes (PBL) were obtained
   from the buffy coat of 15 ml of blood from healthy normal
10 donors. The cells were washed by centrifugation for 5
   min.

      Total cellular RNA was extracted using the UltraSpec
   isolation system (Biotecx, Houston, TX) or Tri-Reagent
15 isolation system (Molecular Research Center, Inc.,
   Cincinnati OH) as described by the manufacturer. For
   UltraSpec, the cells were lysed in 2 ml of UltraSpec RNA
   reagent by repetitive pipetting and placed in ice for 5
   min. Four hundred μl of chloroform was added and mixed
20 rigorously for 15 sec and placed on ice for 5 min. The
   solution was centrifuged at 12,000 X g at 4°C for 15 min.
   The upper phase was transferred into a RNAse-free
   eppendorf tube, 1 volume of isopropanol was added and the
   solution was precipitated at 4°C for 10 min. The tube
25 was centrifuged at 12,000 g at 4°C for 20 min. The
   sample was washed with 70% ethanol, dried, and
   resuspended in 50 μl of DEPC (diethylpyrocarbonate)-
   treated Tris-EDTA (TE) buffer.

30   For Tri-Reagent, the cells were lysed in 1 ml of
   Tri-Reagent by repetitive pipetting and were placed on
   ice for 5 min. Two hundred μl of chloroform was added
   and mixed vigorously for 15 sec and incubated at room
   temperature for 5 min. The solution was then centrifuged
35 at 12,000 X g at 4°C for 15 min. The upper aqueous phase
   was transferred into an RNAse-free eppendorf tube, equal
   volume of isopropanol was then added and the nucleic acid

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was allowed to precipitate at room temperature for 10
min. The tube was then centrifuged as 12,000 X g at 4°C
for 10 min. The sample was washed twice with 70%
ethanol, vacuum-dried, and resuspended in 10mM Tris-HCl
5   with 1 ml EDTA solution (pH 7.4). The concentration of
total RNA was determined using a Beckman
spectrophotometer. One μg of total RNA was used in the
PCR assay to detect mRNA.

10   All extraction procedures for each specimen were
carried out separately in a designated laminar flow hood
under sterile conditions to avoid potential RNA cross-
contamination. PCR reagent set up and post-PCR gel
electrophoresis were carried out in separate rooms to
15   avoid potential RNA cross-contamination.

      (iii)   Oligonucleotide primers and probes

      Oligonucleotide primers were synthesized and
20   purified at the Molecular Biology Institute Core
Facility, UCLA. Oligonucleotide 5-' and 3'-primers for
individual genes were as follows: MAGE-3 primers were
5'-GAAGCCGGCCCAGGCTCG-3' (SEQ ID NO: 1) and
5'-GGAGTCCTCATAGGATTGGCTCC-3'(SEQ ID NO: 2); MUC18
25   primers were 5'-CCAAGGCAACCTCAGCCATGTC-3' (SEQ ID NO: 3)
and 5'-CTCGACTCCACAGTCTGGGACGACT-3'(SEQ ID NO: 4); MUC18
nested primers were 5'-GTCATCTTCCGTGTGCGCCA-3'(SEQ ID NO:
5) and 5'-GTAGCGACCTCCTCAGGCTCCTTAC-3'(SEQ ID NO: 6);
tyrosinase were 5'-TTGGCAGATTGTCTGTAGCC-3' (SEQ ID NO: 7)
30   and 5'-AGGCATTGTGCATGCTGCTT-3'(SEQ ID NO: 8); tyrosinase
nested primers were 5'-GTCTTTATGCAATGGAACGC-3' (SEQ ID
NO: 9) and 5'-GCTATCCCAGTAAGTGGACT-3'(SEQ ID NO: 10); and
p97 primers were 5'-TACCTGGTGGAGAGCGGCCGCCTC-3' (SEQ ID
NO: 11) and 5'-AGCGTCTTCCCCATCAGTGT-3' (SEQ ID NO: 12).
35   The amplification products of MAGE-3, MUC18, MUC18
"nested," tyrosinase, tyrosinase "nested" and p97 were
423, 437, 262, 284, 207 and 286 bp, respectively.

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(iv) Reverse Transcription and Polymerase chain
reaction (RT-PCR)

The RT-PCR assay was performed as previously
5   described with some modifications (Hoon *et al.*, 1993).
An oligo (dT)$_{15}$ primer was employed for the reverse
transcription step, to produce cDNA and guarantee its
amplification over genomic DNA. The reverse
transcription (RT) mixture consisted of 4 μl 25 mM MgCl$_2$,
10   2 μl 10X RT buffer, 4 μl 10 mM dinucleotide triphosphate
mixture, 0.5 μl RNAs in (40 U/μl), 1 μl AMV reverse
transcriptase (9 U/μl), and 1 μl oligo(dT)$_{15}$ primer (1.5
μg/μl). Three μg of sample RNA was added to the RT
mixture and H$_2$0 was added to bring the volume up to 20
15   μl. All reagents were obtained from Promega (Madison,
WI). The reaction was incubated at 42°C for 2 hr, 99°C
for 5 min, and on ice for 5 min.

The PCR mixture consisted of 10 μl 10X PCR buffer
20   (Perkin Elmer Cetus, Norwalk, CT), 8 μl 10 mM dNTPs
mixture, 1 μl 5'-primer (100 pmol/μl), 1 μl 5-' and 3'-
primer, 0.5 μl AmpliTaq 5 U/μl (100 mM Tris-HCl, pH 8.3,
500 mM KCl, 15 mM MgCl$_2$, 0.01% gelatin, Perkin Elmer
Cetus) and 20 μl of RT mixture. Sterile, double-
25   distilled H$_2$0 was added to the mixture to bring it up to
100 μl. The mixture was overlaid with mineral oil. The
PCR conditions were set up as follows: 95°C for 5 min
followed by 95°C for 70 sec, 52°C for 70 sec, 72°C for 70
sec for 40 cycles, and 72°C for 10 min extension time and
30   soaked at 4°C. The PCR reaction was performed in an
OmniGene temperature cycler (Hybaid, Middlesex, England).

To assess nested primers to a particular gene the
PCR mixture after completion through the PCR cycling was
35   added (10 μl) to 10 μl of 10x PCR buffer, 8 μl of 10 mM
dNTPs, 1 μl of 5'-nested primer (100 pmol/μl), 1 μl of
3'-nested primer (100 pmol/μl) and 0.5 μl of AmpliTaq

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polymerase (5 U/μl). The volume of the mixture was
brought up to 100 μl. The PCR cycling was performed as
for the first reaction except the annealing temperature
was 55°C. The preparation of PCR mixture for the
5   temperature cycler was carried out in a designated PCR
room in a specified laminar flow hood.

The PCR amplification product was detected by
electrophoresis on a 2% agarose gel (GIBCO BRL, Grand
10   Island, N.Y.) and visualized by ethidium bromide staining
under ultra violet light. An ϕX174RF DNA/*Hae* III
fragment DNA ladder (BRL) was used as a size reference
marker for all assays.

15   **B.   RESULTS**

The screening process involved examining 10
established melanoma cell lines (10^6 cells/line) and 39
normal PBL (10^7 cells/blood draw) as controls. In Table
20   4, the expression of these markers is shown for melanoma
cells and PBL. A positive reaction was considered as a
visible specific PCR amplification product by gel
electrophoresis stained with ethidium bromide.

25   **Table 4**
                  PCR analysis of melanoma marker genes

Marker gene	Melanoma cell lines	PBL
Tyrosinase	9/10	0/39
p97	10/10	0/39
MAGE-3	8/10	0/39
MUC18	10/10	2/39

RNA was extracted from melanoma and PBL and assessed
for expression of individual markers by PCR. Data
presented as positive cell lines or PBL over total
35   number of specimens assessed. Positive PCR refers to
PCR amplification product assessed by gel
electrophoresis.

- 35 -

All four markers were transcribed in all the
melanoma lines, except for MAGE-3. A melanoma cell line
expressing all four markers would produce cDNA PCR
products of size; 284 base pairs (bp), 286 bp, 423 bp and
5   437 bp (tyrosinase, p97, MAGE-3 and MUC18 respectively)
as observed after electrophoresis through an agarose gel
with ethidium bromide staining and compared with DNA size
markers. In one melanoma cell line, tyrosinase
expression by PCR was negative; however, when nested PCR
10   for tyrosinase was performed tyrosinase gene expression
was detected. There was no detection of melanoma markers
in PBL from 39 normal donors, except two donors which
were positive for MUC18 gene transcription. These
individuals were tested multiple times from separate
15   blood draws and always remained positive for MUC18. This
indicated they were not false positive results due to PCR
contamination or contamination from normal tissue during
blood drawing.

20   In all assays, MUC18 nested primer PCR was
performed; this procedure increased the sensitivity to
allow verification and amplification of weak bands
produced by PCR with only MUC18 primers. Melanoma cell
lines and PBL were tested at least twice to verify
25   specificity. Respective controls in each assay included
samples with positive RNA for the gene being assessed,
PCR reagents and primers without RNA, human tumor cell
lines which were negative for individual gene expression,
and β-actin gene expression.
30

MAGE-1, a gene family member of MAGE-3, also was
tested and found to be transcribed in less than 50% of
the melanoma cell lines. It was decided not to use this
marker for melanoma, since MAGE-3 is more highly
35   expressed in melanomas and MAGE-3 is usually found when
MAGE-1 is expressed (Gaugler et al., 1994). Expression

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of both genes were not detected in PBL from normal
volunteer donors.

EXAMPLE II
Sensitivity of Multiple Melanoma Markers

5

A.   MATERIALS AND METHODS

RNA was isolated and quantitated from melanoma cell
10   lines positive for individual markers.   Specific marker
PCR analysis was then carried out on serial diluted RNA
as described in Example I.

(i)   Southern blot analysis

15

After electrophoresis of PCR amplification products,
agarose gels were transferred overnight onto
nitrocellulose membrane (Schleicher & Schull, Keene,
N.H.) with 20X SSC buffer as previously described.   The
20   cDNA was then UV-crosslinked onto the membrane and
hybridized overnight with a digoxigenin labelled probe
(Morisaki _et al._, 1992).   After hybridization, the
membrane was washed in 2X SSC, 0.1% SDS for 10 min. at
room temperature and then in 0.1X SSC, 0.1% SDS for 30
25   min. at 68°C to remove nonspecific binding (Sambrook
_et al._, 1989).   Specific binding was detected using anti-
digoxigenin, alkaline phosphatase-conjugated antibody as
described by the manufactor (Genius Kit; Boehringer
Mannheim, Indianapolis, IN).   Tyrosinase probes were
30   either prepared, full-length from PCR cDNA products using
the outermost PCR oligonucleotide primers, or 2K bp
probes were _Eco_ R1 digested from plasmids containing the
tyrosinase gene sequence (Kwon _et al._, 1987).   All other
probes used in Southern blotting, were prepared from PCR
35   cDNA products using the outermost oligonucleotide
primers.

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Gel electrophoresis and Southern blotting also was performed automatically using the Automated Electrophoresis System, National Genetics, Inc. and U.S. Patent No. 5,279,721.   See above.

5

B.   RESULTS

In general, all markers could be detected at picogram levels of RNA by visual examination of gel-
10   electrophoresed PCR amplification products stained with ethidium bromide.   RNA from melanoma cell lines were diluted in a series from 1 to 10^{-9} μg and assessed for markers tyrosinase, p97, MUC18, and MAGE-3.   Sensitivity varied for individual lines with different levels of gene
15   expression.   In general, mRNA for p97, MUC18, and MAGE-3 was detected around 10-100 pg by PCR.   Tyrosinase mRNA could be detected at 10-100 fg by PCR.

Specificity of the amplification products was
20   demonstrated by Southern blotting with respective specific probes (tyrosinase, p97).   Sensitivity of the PCR assay could be enhanced 10- to 100-fold using PCR followed by probe blotting.   Nested PCR for tyrosinase enhanced detection levels 10-100 fold above PCR for
25   tyrosinase.   However, nested PCR for MUC18 enhanced results about 10-fold compared to standard PCR for MUC18.

EXAMPLE III
Detection of Melanoma Cells Mixed With PBL _in vitro_
30

A model system mimicking circulating melanoma cells in blood was developed.   In this assay, system 10^7 normal PBL were mixed with serial dilutions of melanoma cells (10^6 to 1 cell) and assessed by PCR for individual gene
35   markers.   The PCR amplification products were then assessed by ethidium bromide staining of gels and by Southern blot analysis.   RT-PCR amplification was also

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performed on RNA extracted from 10^7 PBL and 10^1 melanoma
cells, as controls. Southern blot analysis performed for
tyrosinase verified the specificity of the PCR
amplification product and demonstrated enhanced
5   sensitivity. Materials and methods were as described in
Examples I and II.

Gel electrophoresis or nested primers analysis
demonstrated that melanoma cells could be detected at
10   about 1 cell in 10^7 PBL for tyrosinase, p97 and MUC18.
PBL controls were negative for individual markers in both
gel staining and Southern blot analysis and in both
standard and nested PCR. Specific dilutions of melanoma
cells, were also analysed, in 50 million PBL and
15   demonstrated that about 1-5 melanoma cells could be
detected in 50 million PBL with nested primer tyrosinase
PCR followed by probing with tyrosinase cDNA.

To demonstrate the sensitivity and reproducibility
20   of detecting 1 melanoma cell in 10 million PBL, a Poisson
distribution analysis was carried out. In 8 of 11
samples, a positive PCR amplification product developed
by tyrosinase PCR was detected by gel electrophoresis.
The level of detection was enhanced >90% when tyrosinase
25   nested PCR primers or Southern blot analysis with
tyrosinase probe was performed.

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EXAMPLE IV

Assessment of Circulating Melanoma Cells in Patients Blood

5   A. MATERIALS AND METHODS

(i) Patients

All melanoma patient with complete documented
10   physical and medical histories were accrued from JWCI.
Melanoma patients studied were AJCC (American Joint
Committee on Cancer) stage I, II, III and IV. Patients
assessed were NED (no evidence of clinical disease), AWD
(alive with clinical disease) or EXP (expired during
15   follow-up). The accrual and study of patients was
carried out in a double-blind fashion. The patients'
disease status was not known to the individual running
the PCR assay nor the analyzer of the PCR data. Clinical
disease status was documented at the time of blood
20   drawing and again at 8-15 month follow-up period. PCR
data results were not known to individuals recording the
patient status during the follow-up period.

Fifteen ml of blood was obtained from patients and
25   collected in sodium citrate tubes. All blood was drawn
in the John Wayne Cancer Clinic using the same procedure.
Blood was drawn after written consent was obtained from
the patient. The protocol for the study was approved by
the Saint John's Hospital and John Wayne Cancer Institute
30   Human Subjects Committee. Tubes were centrifuged for 20
min at 2000 x g. The buffy coat was carefully removed
and diluted in double distilled water. The cells were
washed by centrifugation for 5 min. All other materials
and methods were performed as described in Example I and
35   II.

- 40 -

(ii) Protocol

Materials and methods were as described in Examples
I and II. PBL from melanoma patients were examined using
5   an optimized PCR assay detection system. The protocol
was as follows: PCR assays were performed to detect
transcripts of tyrosinase, p97, MAGE-3, and MUC18. All
melanoma patients were subjected to all four tests. If
the sample was negative for tyrosinase or MUC18, nested
10   PCR was performed with respective primers. If the PBL
specimen was negative in the PCR assay for tyrosinase
nested primers and p97 markers, then Southern blot
analysis would be performed with respective probes. PBLs
negative for all the markers and tests were considered as
15   true negatives.

Initially, PBL isolated by Ficoll-hypaque gradient
centrifugation were compared to buffy coat isolated PBL.
In the analysis, buffy coat isolated cells appeared
20   better for the detection of circulating melanoma cells in
blood by PCR.

B.   RESULTS

25   A summary of the analysis of blood specimens by PCR
using multiple markers, as assessed by ethidium bromide
stained gels, is shown in Table 5. The greatest number
of positive patients was observed with MUC18 (73%), with
tyrosinase (59%), p97 (54%) and MAGE-3 (10%) identifying
30   few. Analysis with nested primers of tyrosinase versus
tyrosinase primers significantly increased the number of
positive patients from 2 to 57. Further analysis of p97-
negative and tyrosinase-negative patients with respective
specific probes significantly increased the number of
35   positive patients. The most significant increase was
observed by Southern blotting with the p97 probe. Six

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patients were positive for all four markers. All six
patients were stage IV.

Table 5

**Analysis of melanoma patients using multiple
marker PCR assay**

Assays	Number of patients positive			
	p97	tyrosinase	MAGE-3	MUC18
PCR	16	2	12	80
Nested PCR	-	57	-	87
cDNA blot	49	12	-	-
Total patients	65	71	12	87

Melanoma patients (120) were evaluated. PCR and cDNA
blot refer to assays positive for individual marker
genes. Nested PCR refer to specimens tested negative for
tyrosinase PCR, and + or - for MUC18 PCR that become
positive after nested PCR. cDNA blot refers to patients
tested that were negative for either PCR or nested PCR
and became positive after cDNA blotting.

The PCR analyses were correlated with disease stage
and status (AWD & EXP, NED) of patients. The follow-up
time for clinical status after blood drawing for PCR
analysis was 8-15 months. In the study, there were 4,
18, 32, and 66 Stage I, II, III, and IV patients,
respectively. The majority of the patients in individual
Stages II to IV were PCR positive. Table 6.

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Table 6
PCR positive patients correlation to
Disease status patient status

AJCC Stage	AWD & EXP	NED
I	NP	1/4
II	NP	16/18
III	5/6	23/26
IV	46/48	17/18
Total positive	51/54 (94%)	57/66 (86%)

Values represent patients PCR positive (1 or more markers) over total patients evaluated. NP refers to no patients. AWD & EXP refer to patients AWD and those who were AWD during the blood draw and expired (EXP) during the follow-up period.

The detection of PCR markers was correlated with the Breslow thickness and Clark level of the primary melanoma, after it had been surgically removed. The latter two factors play a role in determining the patients prognosis (Breslow, 1970; Morton et al., 1993). Breslow thickness has been shown to correlate very well with disease progression. Breslow thickness was divided into subgroups of 0.75 mm or less, >0.75 mm to 1.49 mm, ≥1.5 mm to 3.0 mm and >3.0 mm. However, there was no significant correlation of Breslow thickness and detection of PCR markers. Although the majority of the patients were either Clark's level 3 or 4, no significant pattern was observed for Clark's level and number of positive PCR markers. Neither the number of tumor-positive regional lymph nodes nor the sites of distal metastases significantly correlated with the number of positive PCR markers.

The lack of correlation between primary melanoma Breslow thickness and Clark's level with the number of PCR positive markers may be due to the fact that tumor

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progression is no longer dependent on these initial pathological parameters of the primary tumor once it has been removed.

5   **EXAMPLE V**
                          <u>Statistical Analysis</u>

To assess the difference between using tyrosinase alone as a marker and using tyrosinase, MUC18, p97 and
10   MAGE-3 together, a coefficient in level for small sample proportion analysis was performed. Assessment of significance of disease stage to PCR data that was analyzed is summarized below:

15   n = 120   Stage I =   4 NED = 65
                   Stage II = 18 AWD = 38
                   Stage III = 32 EXP = 17
                   Stage IV = 66

20   Of the 120 patients, 49 tested negative for tyrosinase. 42 of these tested positive for at least one of the other three markers (MUC18, P97, MAGE-3). This improvement is statistically significant at the 99% confidence level. It can therefore, be concluded that
25   the four marker PCR assay is more sensitive than the single marker (tyrosinase) assay.

Next, an attempt to correlate a patient's disease stage (I, II, III, or IV) and the number of positive
30   markers (0-4) was undertaken. Table 7 shows the breakdown.

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Table 7

Number of PCR markers correlated to
stage and disease status

Disease stages	Patients Number of positive markers					Total Patients
	0	1	2	3	4	
I	3	0	0	1	0	4
II	2	6	5	5	0	18
III	4	5	15	8	0	32
IV	3	15	23	18	7	66
Total	12	26	43	32	7	120

Positive markers refer to detection of tyrosinase, p97, MUC18 and MAGE-3 by either PCR, nested PCR or Southern blotting.

The results show a positive correlation between stage and the number of positive markers, p = 0.0025, *i.e.*, as stage increases, the proportion of positive markers also seems to increase.

In the follow-up period after blood drawing, patients were divided into those with clinical evidence of disease progression and those with no evidence. The number of patients positive for 0 to 4 PCR markers was correlated to disease progression. The relationship between progression and the number of positive markers also was assessed. Analysis showed that there was a significant correlation (p <0.05) between number of positive markers and disease progression. Table 8.

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Table 8
Number of PCR markers correlated to
progression of disease

	0 pos	1 pos	2 pos	3 pos	4 pos	TOTAL
No Progression	9	13	32	17	2	73
Progression	3	13	11	15	5	47
TOTAL	12	26	43	32	7	120

Thus, although tyrosinase has been used as a marker
in a previous report, the studies disclosed herein
indicate that tyrosinase alone is not always sensitive in
detecting circulating melanoma cells. The use of more
than one marker can verify the presence of occult
melanoma cells and significantly increase the sensitivity
of detecting melanoma cells that express few or no copies
of tyrosinase mRNA. The study demonstrated that using
four markers was significantly better than tyrosinase
alone. In addition, the number of markers detected in
individual patients correlated, significantly, with stage
and disease progression. This higher expression of
individual marker genes indicates, that there is an
increase in the heterogeneity of tumor cells or an
increase in the number of cells in circulation, at
advance stages of disease.

Overall, the level of detection was similar for
tyrosinase and p97 markers. MUC18 marker was the most
frequently detected whereas, MAGE-3 was the lowest.
Although MAGE-3 is expressed in cell lines and biopsies
in higher frequency, the number of mRNA copies in a
single tumor cell is likely to be very low. This may be
related to the state of the cell or clonal phenotype
during circulation in the blood.

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EXAMPLE VI

Detection of β-HCG mRNA Expression in Melanoma Cells

A. MATERIALS AND METHODS

5

(i) Melanoma Cell Lines

Twenty-four melanoma cell lines were established and
characterized at John Wayne Cancer Institute as
10 previously described (Hoon et al., 1991). Cell lines
were cultured and passaged as described in Example I.

(ii) RNA extraction

15   Total cellular RNA was extracted, isolated and
purified using Tri-Reagent according to the
manufacturer's protocol (Molecular Research Center, Inc.
Cincinnati, OH) and described in detail in Example I.
Cells from melanoma lines were lightly trypsinized and
20 collected from tissue culture flasks. Biopsy specimens if
cryopreserved were rapidly thawed and kept in a ice water
bath. Tumor biopsies were kept in a ice water bath when
being minced. All RNA extraction was carried out in a
sterile designated laminar flow hood with RNase free
25 labware. Purified RNA was quantitated and assessed for
purity by UV spectrophotometry.

(iii) Oligonucleotide primers and Probes

30   Oligonucleotide primers were synthesized and
purified at the Molecular Biology Institute Core
Facility, UCLA. The β-HCG primer sequences were as
follows: 5' primer was 5'-ATGCCACCCTGGC TGTGGAGAA-3' (SEQ
ID NO: 13) and the 3' primer was
35 5'-GGGAGTCGGGATGGACTTGGAA-3' (SEQ ID NO: 14). The RT-PCR
cDNA product was 367 bp. The 5'primer has only one
mismatch with the β-luteinizing hormone (LH, see below)

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while the 3' primer is homologous to both β-HCG AND β-LH
coding regions. A full-length, PCR product, amplified
from β-HCG DNA, was used a probe for Southern blot
analysis.

5

The sequences of α-HCG primers were derived from the
GenBank; 5'-AAAGGAGCGCCATGGATTAC-3'(SEQ ID NO: 15); and
3' primer, 5'-CCATTACTGTGACCCTGTTA-3'(SEQ ID NO: 16).
The α-HCG PCR cDNA product was 297 bp. The primer

10 sequences for β-HCG/LH receptors were 5'primer, 5'-
CCCGATGTGCTCCTGAACCAGA-3'(SEQ ID NO: 17); and 3'primer,
5'-GCTGACACCGACAAGGGGCAA-3' (SEQ ID NO: 18). The RT-PCR
cDNA product for β-HCG/LH receptors was 496 bp. The
β-actin primer sequences were as follows: 5' primer was

15 5'-GGAGCAATGATCTTGATCTTC-3' (SEQ ID NO: 21) and the 3'
primer was 5'-CCTTCCTGGGCATGGAGTCCTG-3' (SEQ ID NO: 22).
The RT-PCR product was 201 bp. The tyrosinase and MAGE-3
primers were the same as described in Example I.

20 .   *(iv) RT-PCR assay*

The RT-PCR assay was carried out as previously
described (Morisaki *et al.*, 1992, and in Example I).
Briefly, reverse transcription was carried out with oligo

25 (dT)$_{15}$ primer and AMV reverse transcriptase with 5 ug of
RNA and incubated for 2 hr at 42°C and 99°C for 5 min.
The RT-PCR conditions were set up as follows: 95°C for 5
min followed by 95°C for 1 min, 65°C for 1 min, 72°C for
1 min, and 72°C for 10 min for final primer extension

30 sequence and performed in an OmniGene thermocycler
(Hybaid, Middlesex, England).

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(v)   Restriction digestion

β-HCG is a gonadotropin hormone composed of an α-
and β-subunit (Giuliano, *et al.*, 1995; Fiddes *et al.*,
5   1979; Boorstein *et al.*, 1982). The amino acid sequence
of α-HCG is essentially indistinguishable from those of
the other human gonadotropin hormones, such as follicle-
stimulating, luteinizing, and thyroid-stimulating
hormones (Fiddes *et al.*, 1979; Pierce *et al.* 1981).
10   However, the β-HCG subunit is different amongst the other
hormone subunits except for the β-LH subunit; they share
82% common amino acid sequence (Talmadge *et al.*, 1984).
The ß-subunit of HCG to date has been shown to consist of
cluster of 6 related genes linked closely to the ß-LH
15   single gene (Bo *et al.*, 1992).   Since the β-HCG and β-LH
are highly homologous it is not possible to design a
primer sequence absolutely specific to β-HCG.

However, the β-HCG PCR cDNA product has a unique *Sty*
20   I restriction site that is not present in the β-LH PCR
cDNA product. Digestion of PCR products with this enzyme
allows β-HCG to be destinguished from β-LH. RT-PCR cDNA
product was incubated with 10X NEBuffer 3 (New England
BioLabs, Beverly, MA) and *Sty* I (10 U/ml) (New England
25   Biolabs) and the mixture was incubated overnight at 37°C.
The endonuclease digested product mixture was run on a 2%
agarose gel and stained with Etbr. β-HCG RT-PCR cDNA
product digested with *Sty* I produces a 271 and 96 bp
band. If no digestion occurred the reaction was repeated
30   at least twice to confirm.

(vi) Southern blotting

RT-PCR cDNA products run on a 2% agarose gel were
35   denatured and transferred overnight onto nylon membrane
(Micron Separations, Inc.) as previously described in
Example II.   β-HCG cDNA probe was prepared by PCR,

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purified and digoxigenin labelled as described in
Example II.

B.   RESULTS

5

        Assessment of β-HCG expression in cells by molecular
techniques has been difficult because of the sequence
homologies of both α and β subunits to related hormones.
The terminal end of the β-chain subunit chain was chosen
10   as a target for RT-PCR since it had the most significant
differences compared to other related hormone β-chain
subunits.

        Initially 24 established human melanoma cell lines
15   derived from different anatomical sites were assessed to
determine if they expressed β-HCG chain.   Oligo dT$_{(15)}$
priming was carried out to assess only poly A mRNA of
β-HCG. Of the 24 cell lines tested by RT-PCR, 16 of 24
produced a specific cDNA product of the correct size (367
20   bp) as verified by Etbr gel staining.

        β-actin was run on all samples as an internal
control to verify RNA yield and efficiency of the RT-PCR
assay.   Each assay had a negative control consisting of
25   RT-PCR reagents alone without RNA and a positive control
for β-HCG. Southern blot analysis of PCR cDNA product
with the β-HCG probe showed that three of the cell lines
negative by Etbr staining had a specific cDNA band.
However, one cell line in which the Etbr staining was
30   questionable showed no specific band on Southern blot
analysis. Overall 18 out of 24 cell lines were positive
(75%) for β-HCG marker expression.

        To further verify β-HCG marker expression,
35   endonuclease restriction digestion with Sty I was carried
out on the RT-PCR cDNA products. All cDNA products
digested, produced two bands, 271 bp and 96 bp as

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observed on Etbr gels, indicative of β-HCG marker. These
digested products were further verified by Southern blot
analysis with β-HCG cDNA probe.

EXAMPLE VII
Detection of β-HCG mRNA Expression
in Melanoma Tumor Biopsy Specimens

A.   MATERIALS AND METHODS

(i)   Melanoma Tumor Biopsy Specimens and Blood
Preparation

Melanoma tumor biopsy specimens that were defined by
histopathology as malignant melanoma were assessed.
Melanoma biopsies were obtained from primary lesions and
from multiple anatomical sites of metastatic lesions from
different patients. Specimens were immediately frozen or
processed as received from the operating room. In this
study liquid nitrogen cryopreserved and fresh tumor
biopsies from surgery were assessed. On obtaining
melanoma biopsies non-melanoma tissue was carefully
dissected away from normal tissue under sterile
conditions in a laminar flow hood.

PBL were obtained from 25 normal male and female
volunteer donors and the buffy coat was collected for RNA
isolation as described in Example I. All other
techniques including RT-PCR, Southern blotting and
restriction enzyme digests were as described in
Example VI.

Normal axillary lymph node tissue that was assessed
as histopathology negative for tumor was obtained from
melanoma and breast cancer patients undergoing elective
surgery. Axillary lymph nodes were assessed by

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histopathology for malignancy by standard conventional
hematoxylin and eosin (H & E) staining.

B.   RESULTS

.5

Melanoma primaries and metastases have been shown to
contain infiltrating immune cells (Cochran and Hoon,
1987). To be certain that β-HCG mRNA was being expressed
by the tumor cells and not a product of infiltrating
10   lymphoid cells, peripheral blood lymphocytes were also
analysed for expression of β-HCG mRNA. PBL from 25
normal volunteer donors were analysed by RT-PCR but no
evidence of β-HCG expression was observed, even after
Southern blot analysis (except in one individual who was
15   positive in a second blood draw).

, Five lymph nodes from two breast and melanoma
patients, were found to be negative by H & E staining and
by RT-PCR and Southern blotting.
20

Both cryopreserved and fresh biopsy tissue were
anaylsed by histopathology and by RT-PCR, restriction
digestion and Southern blotting. Out of 40 patients, 38
were identified, as melanoma positive by histopathology,
25   while 16 were identified as positive by RT-PCR for β-HCG
marker. In other words, an estimated 42% of melanoma
biopsies were β-HCG positive. All specimens that were
found to be melanoma negative by histopathology, were
also negative for β-HCG marker expression. The detection
30   of β-HCG mRNA was much weaker in melanoma biopsy tissues
as compared to melanoma cell lines. This detected lower
gene activity may be due to the heterogeneity of tumors,
variability of host physiologic regulation of β-HCG, or
simply the dilution of RNA by normal cell infiltrate.
35

There was no significant difference in β-HCG mRNA
detection between cryopreserved and fresh biopsy

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specimens. β-actin expression was detected in all
specimens, thus verifying the integrity of the mRNA and
the PCR assay. α-HCG subunit expression in five β-HCG
positive melanoma cell lines and five melanoma biopsies
5   was also analyzed. However, no α-HCG expression was
detected by RT-PCR even when followed by Southern blot
analysis.

EXAMPLE VIII

10   **A Comparison of β-HCG mRNA Expression with**
Other Melanoma Markers

A.   MATERIALS AND METHODS

15   *(i)   Surgical Specimens*

Axillary lymph node tissue was taken after elective
surgery of TDLN from seven melanoma patients. TDLN were
assessed by histopathology for malignancy by standard
20   conventional hematoxylin and eosin (H & E) staining.
β-HCG mRNA expression was compared with tyrosinase and
MAGE-3 mRNA expression by RT-PCR. All other materials
and methods were as described in Example VI.

25   **B.   RESULTS**

Out of eight tumor-draining lymph nodes (TDLN) (from
seven melanoma patients) five were positive for β-HCG
expression, six for tyrosinase and three for MAGE-3. In
30   two patients, none of the markers were detected.

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Table 9
Analysis of β-HCG expression in melanoma TDLN

TDLN	Path	β-actin	β-HCG EtBr	β-HCG S. Blot	β-HCG Sty I	Tyr	MAGE3
1	+	+	-	+	+	+	-
2	+	+	-	+	+	+	-
3	+	+	-	-		+	+
4	+	+	+	+	+	+	+
5	+	+	-	-		-	-
6	+	+	+	+	+	+	+
7a	+	+	+	+	+	+	-
7b	-	+	-	-		-	-

TDLN refer to individual patient nodes examined (a and b refer to two separate nodes). Pathology refers to the hematoxylin and eosin staining histopathology analysis of lymph node sections. + refers to presence of melanoma metastases and - refers to no metastases. RT-PCR analysis detected by Etbr and Southern blot is indicated as + or -. Tyr refers to RT-PCR analysis by tyrosinase primers followed by nested tyrosinase RT-PCR if negative.

In conclusion, β-HCG is a useful addition to the group of melanoma markers described in Examples I through V. The frequency of expression of β-HCG mRNA in melanoma appears to be similar to that of the melanoma tumor antigens MAGE-3 and MAGE-1.

EXAMPLE IX
Detection of GalNAc mRNA Expression
in Melanoma Cells and Biopsies

A.   MATERIALS AND METHODS

        (i)   *Melanoma cell lines* and *Surgical Specimens*

        Melanoma cell lines were all established at JWCI and grown as described in Example I.   20 melanoma tumor

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biopsy specimens were obtained as described in Example
VII.   RNA extraction and RT-PCR assay was as described in
Example BI.

5   (iv) Oligonucleotide primers and Probes

          Oligonucleotide primers were synthesized and
purified at the Molecular Biology Institute Core
Facility, UCLA.   The GalNAc primers used were: 5'-
10   CCAACTCAACAGGCAACTAC-3' (SEQ ID NO: 19) and 3'
GATCATAACGGAGGAAGGTC-3' (SEQ ID NO: 20).   cDNA probes,
amplified by PCR with these primers, were used in
Southern blotting, which was performed as described in
Example II.   The tyrosinase and MAGE-3 primers were the
15   same as described in Example I and β-HCG primers were the
same as described in Example VI.

Table 10

GalNAc expression in melanoma biopsies and cell lines

SPECIMENS	GalNAc EXPRESSION		
BIOPSIES# METASTASES			
19	-		
25	-		
68	-		
100	+		
102	+		
178	-		
221	-		
224	-		
246	+		
250	-		
260	-		
261	-		
287	-		
292	+		
295	+		
301	+		
351	+		
361	+		
380	+		
443	+		
MELANOMA CELL LINES			
MATT	+		
M101	+		
M12	+		
M24	+		
M10	+		
M18	+		
MKN	+		

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Table 10 (continued)

SPECIMENS	GalNAc EXPRESSION		
MHL	+		
MCE	+		
MKE	+		
MELL	+		
MMAC	+		
MF	-		
M18	+		
PERIPHERAL BLOOD LYMPOCYTES			
DONOR #1	-		
DONOR #10	-		
DONOR #12	-		
DONOR #13	-		
DONOR #322	-		
DONOR #323	-		
DONOR #324	-		
DONOR #325	-		
DONOR #326	-		
DONOR #338	-		
DONOR #339	-		
DONOR #340	-		
DONOR #342	-		
DONOR #343	-		
NORMAL LYMPH NODE #349	-		
NORMAL LYMPH NODE #364	-		

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B.   RESULTS

As shown in Table 7, detection of GalNAC mRNA was
succesfully detected in 13 out of 14 melanoma cell lines
5   and 10 out of 20 biopsy specimens. Furthermore, no
GalNAc marker expression was observed in normal lymph
nodes or PBL. These are similar results to those found
for β-HCG and MAGE-3 in previous examples. Indicating
that GalNAc mRNA expression is another marker which may
10   be utilized for the detection of melanoma and metastases.

Amplification of GalNAc mRNA is an indicator of
gangliosides, GM2 and GD2, expression. Direct detection
of GM2 and GD2 in occult metastases and small tumor
15   lesions such as melanoma primaries is very difficult and
often impractical when using standard biochemical
methods. Monoclonal antibodies to gangliosides are
available but often cross-react with other carbohydrate
structures and therefore are not reliable and do not
20   represent absolute ganglioside expression (Hoon et al.,
1993).

Detection of tumor cells with the marker GalNAc by
RT-PCR provides a novel approach to detect metastatic
25   melanoma and breast cancer cells in blood or fluids that
would not be possible by current biochemical or
immunological techniques.

EXAMPLE X

30   <u>Detection of β-HCG mRNA Expression in Breast Cancer Cells</u>

A.   MATERIALS AND METHODS

(i) Breast cell lines

35

The established breast cancer cell line JWCI BM-1
was developed from a primary invasive ductal carcinoma

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tumor and characterized as a breast cancer line at the
John Wayne Cancer Institute. Breast cell lines MDA-MB-
231, MCF-7, BT-549, T-47D and BT-20 were obtained from
the American Type Culture Collection (ATCC, Rockville,
5   MD) and were cultured according to instructions provided.
The 734B line is an established subclone of MCF-7. Cells
were grown in 10% fetal calf serum (heat-inactivated)
RPMI 1640 (Gemini Bioproducts, Calabasas, CA) plus
penicillin and Streptomycin (GIBCO, Long Island, NY) in
10   T75 cm^2 flasks. Adherent cell lines were routinely
passaged by trypsinization every 3-4 days. When cell
lines attained 75-85% confluency they were used for PCR
analysis.

15   *(ii) RNA preparation*

Tri-Reagent (Molecular Research Center, Inc.
Cincinnati, OH) was used to isolate total RNA from the
cell lines and surgical specimens, following the
20   manufacturer's instructions and described in Example I.
One μg of total RNA was used in the PCR assay to detect
β-HCG mRNA. Oligonucleotide primers and probes were as
described in Example VI.

25   *(iii) RT-PCR*

Reverse transcription was as described in Example I,
using oligo (dT)$_{15}$ and oligo nucleotides as described in
Example VI.
30

The PCR mixture was also as described in Example I
and incubated in an OmniGene temperature cycler (Hybaid,
Middlesex, England) at 95°C for 3 min for 1 cycle; 95° C
for 1 min, 65°C for 1 min, 72°C for 1 min for 30 cycles;
35   and 72°C for 10 min. The PCR cDNA products were assessed
in a 2% agarose gel containing ethidium bromide. A 100
bp DNA ladder (GIBCO BRL Life Technologies Inc.,

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Gaithersburg, MD) was used as a bp reference marker.
Restriction enzyme digestion and Southern blot analysis
were performed as previously described in Example VI.

5   *(iv) β-HCG protein expression in breast cancer cells*

        Breast cancer cells from individual cell lines were
seeded in 12 well tissue culture microplates at 2 million
cells/well in 1.5 ml of RPMI 1640 without fetal calf
10   serum and cultured at 37°C in a tissue culture incubator
for 24 hr. Supernatant was harvested and concentrated 10
times to a volume of 150 μl using Centricon 10
concentrators (Amicon Division, W.R. Grace & Co.,
Beverly, MA). β-HCG in the supernatant was measured
15   using a total β-HCG Quantitative Test kit (Medix Biotech
Inc., Foster City, CA), following the manufacturer's
instructions. Supernatant samples were tested in
duplicate with a standard reference for each assay. The
ELISA reaction was read at 490 nm using a Vmax kinetic
20   microplate reader (Molecular Devices Corp., Palo Alto,
CA).

        B.   RESULTS

25   *(i)   β-HCG mRNA expression in breast cell lines*

        All seven breast cancer cell lines were found to
express β-HCG mRNA. A positive result was indicated by a
367 bp cDNA band as detected by Etbr-staining and
30   Southern blot analysis. To confirm the identity of the
amplified PCR cDNA product, samples were digested with
endonuclease *Sty I*. All seven β-HCG PCR products were
cleaved by *Sty I* to produce bands of 271 and 96 bp on
Etbr gels, thus confirming β-HCG mRNA expression. As a
35   negative control, PBL from 25 normal (male and female)
volunteers were examined. None of the control specimens

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were shown to be positive for β-HCG marker expression by PCR and Southern blotting.

5   The expression of α-HCG mRNA was also examined by PCR in breast cell lines. In cell lines MDA-MB-231, JWCI BM-1, and T-47D, α-HCG mRNA expression was detected by Etbr-stained gel electrophoresis. The α-subunit detected could be HCG or other related hormones since they all share a high degree of similarity in α-subunit. For this 10   reason α-HCG as a cancer marker, is not practical.

(ii) β-HCG protein expression in breast cancer cell lines

15   Out of the seven breast cell lines expressing β-HCG mRNA, only three cell lines (MDA-MB-231, T47-D, JWCI BM-1) secreted detectable levels of β-HCG protein as analysed by ELISA (0.15 mIU, 0.15 mIU, 0.1 mIU/2 x 10^6 cells, respectively). The cell lines, producing β-HCG 20   were those that were positive for α-HCG mRNA expression.

Breast cancer lines were also analysed for β-HCG receptor mRNA. Human β-HCG/LH receptor genes cloned recently, have been shown to possess a high dgree of 25   similarity (Minegish et al., 1990). All cell lines were positive for β-HCG/LH receptor mRNA expression. To evaluate whether β-HCG/LH receptor could be used as a marker for metastatic breast cancers, PBL from six normal volunteer donors (male and female) was analysed for the 30   corresponding mRNA using the primers as described in Example VI. All donors expressed β-HCG/LH receptor mRNA indicating that the β-HCG/LH receptor is not a reliable marker for detecting breast cancer cells in blood or lymph nodes.

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EXAMPLE XI
Sensitivity of β-HCG Marker

A. **MATERIALS AND METHODS**

5

   (i) PCR detection sensitivity

   The sensitivity of the PCR assay to detect β-HCG
mRNA in breast cancer cells was assessed by the following
10   methods:

   (a)   RNA was isolated from MDA-MB-231 cells and
serially diluted from 1 to 10^{-6} μg, and then analysed by
the RT-PCR assay.

15

   (b)   Cell suspension of MDA-MB-231 cells were
prepared and diluted with PBL to produce an *in vitro*
model occult carcinoma cells in lymph nodes. 10^7 PBL
were mixed with a variable number of cancer cells ranging
20   from 1 to 10^5.   Total RNA was then isolated from the
mixtures and analysed by the RT-PCR assay and observed on
an Etbr gel along side a positive (10^6 MDA-MB-231 cells)
and negative (10^7 PBL) control.   All other method were as
described in Example X.   The PBL were obtained from
25   normal volunteers in which PCR analysis had shown no
presence of β-HCG mRNA.

B.   **RESULTS**

30   RT-PCR results of a series of diluted RNA isolated
from MDA-MB-231 was determined on Etbr agarose gels.
β-HCG marker was detected from as little as 10^{-5} ug RNA.
This detection was enhanced ten fold by Southern
blotting, enabling β-HCG mRNA expression detection from
35   as little as 10^{-6} μg of RNA.

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Employing the *in vitro* model it was shown that one
breast cancer cell, determined by the amplification of
β-HCG marker, could be detected up to among 10^7 PBL.

5

EXAMPLE XII

**Detection of β-HCG mRNA Expression in
Breast Cancer Specimens**

10   **A.   MATERIALS AND METHODS**

(i)   Surgical Specimens

Thirty-one lymph nodes were collected from 18
15   patients (13 invasive ductal carcinoma, 4 invasive
lobular carcinoma, and one *in situ* carcinoma) who were
undergoing mastectomy with axillary lymphadenectomy for
clinically early stage breast cancer. Nodes that were
only logistically practical for cutting, for conventional
20   pathological diagnosis and for archive fixation were
obtained for RT-PCR. Patients ranged in age from 37 to
73 years old. In order to compare the results of PCR with
histological analysis, the lymph nodes were divided into
two, one half was analyzed by PCR and the other by
25   histopathological H & E staining of serial sections.

For a negative control, blood was obtained from 25
normal volunteer donors (both male and female). All
further materials and methods were as described in
30   previous examples.

B.   RESULTS

Table 11 shows the results of β-HCG mRNA expression
35   in TDLN. Two TDLN from eleven patients (patient G to Q)
and three TDLN from one patient were analysed. Five of
the TDLNs were found to be negative by conventional H & E

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staining, were found to be positive for β-HCG marker
expression in the PCR assay (No. 4, 16, 19, 23, and 24).
If the RT-PCR assays were found to be negative, Southern
blot analysis was subsequently performed.

5

Overall, 9 out of 31 TDLN were found to be negative
by H & E staining and PCR with or without Southern
blotting. Four of the TDLN which were found to be
negative by both PCR and histological examination, were
10   subsequently found to be positive following Southern blot
analysis (No. 12, 13, 15, and 22). All 367 bp β-HCG PCR
cDNA products detected by PCR or Southern blotting were
digested by restriction enzyme Sty I. There were no TDLN
found to be positive by histological examination, but
15   found negative by PCR or PCR and by Southern blotting.

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Table 11
Detection for β-HCG expressing tumor cells in breast TDLN

Specimen number	Patient	Tumor histology	Pathology	PCR	Southern blot
1	A	lobular	+	+	
2	B	ductal	+	+	
3	C	ductal	+	+	
4	D	insitu	-	+	
5	E	lobular	+	+	
6	F	ductal	+	+	
7	G	ductal	-	-	-
8			-	-	-
9	H	ductal	-	-	-
10			+	+	
11	I	ductal	-	-	-
12			-	-	+
13	J	ductal	-	-	+
14			-	-	-
15	K	ductal	-	-	+
16			-	-	+
17	L	ductal	-	-	-
18			+	+	+
19	M	ductal	-	+	
20			+	+	
21	N	ductal	-	-	-
22			-	-	+
23	O	ductal	-	+	
24			-	+	
25	P	ductal	+	+	
26			+	+	
27	Q	ductal	-	-	-
28			-	-	-
29	R	lobular	+	+	

Table 11 (continued)

Specimen number	Patient	Tumor histology	Pathology	PCR	Southern blot
30			+	+	
31			+	+	

Individual patients are labelled as A - R and numbers
refer to individual TDLN. Tumor histology represents H
& E staining diagnosed pathology. Pathology refers to
diagnoses of + or - for breast cancer metastases. PCR
results are indicated as + or - on Etbr gel
electrophoresis analysis. Specimens negative for RT-PCR
assay were subsequently Southern blotted with β-HCG
cDNA probe. Southern blot analysis is indicated as + or
-.

EXAMPLE XIII

Detection of Breast Cancer Cells by Multiple Markers

A.   MATERIALS AND METHODS

(i) Breast cell lines and Surgical Specimens

The breast cell line MDA-MB-231, MCF-7, BT-549, T-
47D and BT-20 were obtained from the American Type
Culture Collection (ATCC, Rockville, MD) and were
cultured according to instructions provided. The 734B
line is an established subclone of MCF-7. Cells were
grown as described in Example X. 11 breast cancer
biopsies were extracted as described in Example XII. RNA
extraction and RT-PCR assay were as described in
Example X.

(ii) Oligonucleotide primers and Probes

Oligonucleotide primers were synthesized and
purified at the Molecular Biology Institute Core
Facility, UCLA. The MAGE-1 primer sequences were as
follows: 5' primer was 5'-GCTGGAACCCTCACTGGGTTGCC-3' (SEQ
ID NO: 23) and the 3' primer was

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5'-CGGCCGAAGGAACCTGACCCAG-3' (SEQ ID NO: 24). The RT-PCR
cDNA product was 421 bp. The tyrosinase and MAGE-3
primers were the same as described in Example I, β-HCG
primers were the same as described in Example VI and
5   GalNAc primers were the same as described in Example IX.
cDNA probes, amplified by PCR with these primers, were
used in Southern blotting, which was performed as
described in Example II.

10   B.   RESULTS

     Multiple markers were used to analyse breast cancer
cells and breast cancer biopsy specimens by RT-PCR and
Southern blotting. Tables 12 and Table 13 shows the
15   results. All breast cancer cells were positive for at
least five, out of the six, markers. For the biopsy
specimens, at least one of the markers were detected from
all samples. None of the markers alone would have been
able to detect cancer cells in all of the specimens.
20   This variation in marker detection reflects the
heterogeniety of tumor cells. In conclusion, multiple
markers are more sensitive to detection of breast cancer
than any one marker.

25   Table 12
          Analysis of Markers in Breast Cancer Cell Lines

	MAGE3	MAGE 1	MUC18	p97	GalNAc	β-HCG
BT20	+	+	+	- -	+	+
BT549	+	- -	+	+	+	+
902P	+	+	+	+	+	+
T47D	+	+	+	- -	+	+
734B/24	+	+	+	+	+	+
231/45	- -	+	+	+	+	+
MCF7	+	+	+	+	+	+

35   +/-: RT-PCR or Southern Blotting

Table 13

Analysis of Markers in Breast Cancer Biopsies

Biopsy tumor or number	GalNAc	β-HCG	MUC 18	P-97	MAGE-3	MAGE-1
0350T2A122794	+	+	+	--	--	+
0424T2A011795	--	--	--	--	+	--
0433T2A011895	--	+	+	--	+	--
044T2A012095	--	--	--	--	+	--
0460T2A012695	--	+	+	--	+	+
0498T2K020395	--	--	+	--	--	--
0500T2K020695	--	--	+	+	+	--
0506T2A020795	+	+	+	+	--	+
0520T2A020995	--	--	+	--	--	+
0522T2A020995	--	--	+	--	--	--
0525T2A020995	--	--	+	+	--	--

+/-: RT-PCR and Southern Blotting

        Previous PCR studies have not analyzed large numbers
of patients with different clinical stages of melanoma or
breast cancer. This is important in evaluating the
sensitivity and clinical significance of the assay.
Furthermore, this information is useful in staging
disease into clinical subgroups, in particular,
identifying subgroups of patients that need more
intensive therapeutic intervention. For example, in NED
patients with circulating tumor cells, immediate
therapeutic intervention may be a very efficacious means
of controlling potential tumor progression and, thus,
preventing clinical disease. The detection of
circulating cancer cells may also prove useful for
monitoring a patient's response to operative and adjuvant
therapies.

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Applying a multiple melanoma marker method to the evaluation of circulating cancer cells also provides information about the tumor's phenotype. Identification of specific tumor-associated antigen(s) permits the
5   rational use of specific immunotherapy protocols such as monoclonal antibodies and cancer vaccine (Hoon et al., 1993). The PCR assay also provides a rapid monitoring system as a follow-up to determine if a specific therapy is effective.
10

While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the composition, methods and
15   in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted
20   for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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5   supplementary to those set forth herein, are specifically
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cancer. *Cancer*, **73**:2745-2752, 1994.

- 84 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

     (i) APPLICANT:
         (A) NAME: John Wayne Cancer Institue
         (B) STREET: 2200 Santa Monica Boulevard
         (C) CITY: Santa Monica

10
         (D) STATE: California
         (E) COUNTRY: United States of America
         (F) POSTAL CODE (ZIP): 90404

         (A) NAME: National Genetics Institute

15
         (B) STREET: 5839 Green Valley Circle, Suite 104
         (C) CITY: Culver City
         (D) STATE: California
         (E) COUNTRY: United States of America
         (F) POSTAL CODE (ZIP): 90230

20

     (ii) TITLE OF INVENTION: DETECTION OF MELANOMA OR
                            BREAST METASTASES WITH A
                            MULTIPLE MARKER ASSAY

25   (iii) NUMBER OF SEQUENCES: 24

     (iv) COMPUTER READABLE FORM:
         (A) MEDIUM TYPE: Floppy disk
         (B) COMPUTER: IBM PC compatible

30
         (C) OPERATING SYSTEM: PC-DOS/MS-DOS
         (D) SOFTWARE: PatentIn Release #1.0, Version
                      #1.30 (EPO)

     (v) CURRENT APPLICATION DATA:

35   APPLICATION NUMBER: UNKNOWN

     (vi) PRIOR APPLICATION DATA:

- 85 -

(A) APPLICATION NUMBER: USSN 08/406,307

(B) FILING DATE: 17-MAR-1995

5   (2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

10   (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

15   GAAGCCGGCC CAGGCTCG   18

(2) INFORMATION FOR SEQ ID NO: 2:

20   (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GGAGTCCTCA TAGGATTGGC TCC   23

30

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

35   (B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

- 86 -

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCAAGGCAAC CTCAGCCATG TC   22

5

        (2) INFORMATION FOR SEQ ID NO: 4:

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 25 base pairs
10   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:
15

CTCGACTCCA CAGTCTGGGA CGACT   25

        (2) INFORMATION FOR SEQ ID NO: 5:
20

            (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
25   (D) TOPOLOGY: linear

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GTCATCTTCC GTGTGCGCCA   20

30

        (2) INFORMATION FOR SEQ ID NO: 6:

            (i) SEQUENCE CHARACTERISTICS:
35   (A) LENGTH: 25 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

5   GTAGCGACCT CCTCAGGCTC CTTAC   25

(2) INFORMATION FOR SEQ ID NO: 7:

10   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

15

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

     TTGGCAGATT GTCTGTAGCC   20

20

     (2) INFORMATION FOR SEQ ID NO: 8:

          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs
25   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
30
     AGGCATTGTG CATGCTGCTT   20

     (2) INFORMATION FOR SEQ ID NO: 9:
35
          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 20 base pairs

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(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

5   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GTCTTTATGC AATGGAACGC   20

10   (2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 20 base pairs
     (B) TYPE: nucleic acid
15   (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

20   GCTATCCCAG TAAGTGGACT   20

(2) INFORMATION FOR SEQ ID NO: 11:

25   (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 24 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: single
     (D) TOPOLOGY: linear

30
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TACCTGGTGG AGAGCGGCCG CCTC   24

35

(2) INFORMATION FOR SEQ ID NO: 12:

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              (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 20 base pairs
                   (B) TYPE: nucleic acid
                   (C) STRANDEDNESS: single
5   (D) TOPOLOGY: linear

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

     AGCGTCTTCC CCATCAGTGT   20
10

     (2) INFORMATION FOR SEQ ID NO: 13:

              (i) SEQUENCE CHARACTERISTICS:
15   (A) LENGTH: 22 base pairs
                   (B) TYPE: nucleic acid
                   (C) STRANDEDNESS: single
                   (D) TOPOLOGY: linear

20   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

     ATGCCACCCT GGCTGTGGAG AA   22

25   (2) INFORMATION FOR SEQ ID NO: 14:

              (i) SEQUENCE CHARACTERISTICS:
                   (A) LENGTH: 22 base pairs
                   (B) TYPE: nucleic acid
30   (C) STRANDEDNESS: single
                   (D) TOPOLOGY: linear

         (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

35   GGGAGTCGGG ATGGACTTGG AA   22

- 90 -

(2) INFORMATION FOR SEQ ID NO: 15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
5   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:
10

AAAGGAGCGC CATGGATTAC   20

(2) INFORMATION FOR SEQ ID NO: 16:
15

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
20.   (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCATTACTGT GACCCTGTTA   20
25

(2) INFORMATION FOR SEQ ID NO: 17:

    (i) SEQUENCE CHARACTERISTICS:
30   (A) LENGTH: 22 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCCGATGTGC TCCTGAACCA GA   22

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(2) INFORMATION FOR SEQ ID NO: 18:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GCTGACACCG ACAAGGGGCA A   21

(2) INFORMATION FOR SEQ ID NO: 19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCAACTCAAC AGGCAACTAC   20

(2) INFORMATION FOR SEQ ID NO: 20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GATCATAACG GAGGAAGGTC   20

(2) INFORMATION FOR SEQ ID NO: 21:

          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 21 base pairs
5   (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
10

     GGAGCAATGA TCTTGATCTT C   21

     (2) INFORMATION FOR SEQ ID NO: 22:
15

          (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 22 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
20   (D) TOPOLOGY: linear

          (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

     CCTTCCTGGG CATGGAGTCC TG   22
25

     (2) INFORMATION FOR SEQ ID NO: 23:

          (i) SEQUENCE CHARACTERISTICS:
30   (A) LENGTH: 23 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

35   (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

     GCTGGAACCC TCACTGGGTT GCC   23

- 93 -

(2) INFORMATION FOR SEQ ID NO: 24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 22 base pairs
5   (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
10

CGGCCGAAGG AACCTGACCC AG   22

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CLAIMS:

1.   A method for detecting melanoma or breast cancer
cells in a biological sample comprising the step of
5   amplifying at least two nucleic acids in said sample,
said nucleic acids being melanoma or breast cancer
markers.

10   2.   The method of claim 1, comprising the steps of:

(a)   extracting RNA from said sample;

(b)   contacting said RNA with a primer pair that
15   hybridize to one of said melanoma or breast
cancer marker nucleic acids;

(c)   amplifying said melanoma or breast cancer
marker nucleic acids to produce an
20   amplification product;

(d)   detecting said amplification product; and

(e)   repeating steps (b), (c), (d) and (e) with a
25   primer pair that hybridize to at least one of
the other said melanoma or breast cancer marker
nucleic acids.

30   3.   The method of claim 2, further comprising the step
of preparing at least two pairs of primers complementary
to regions of said melanoma or breast cancer marker
nucleic acids.

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4. The method of claim 3, wherein primer pairs for at
least three melanoma or breast cancer markers are
employed.

5

5. The method of claim 4, wherein primer pairs for at
least four melanoma or breast cancer cell markers are
employed.

10

6. The method of claim 5, wherein primer pairs for at
least five melanoma or breast cancer markers are
employed.

15

7. The method of claim 6, wherein primer pairs for at
least six melanoma or breast cancer cell markers are
employed.

20

8. The method of claim 7, wherein primer pairs for at
least seven melanoma or breast cancer cell markers are
employed.

25

9. The method of claim 2, wherein said melanoma or
breast cancer cell markers are selected from the group
comprising; tyrosinase, MAGE-3, MUC18, p97, MAGE-1,
GalNAc and β-HCG.

30

10. The method of claim 2, wherein said amplification is
polymerase chain reaction.

35

11. The method of claim 10, wherein said polymerase
chain reaction is nested.

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12.   The method of claim 2, wherein said RNA is total
cellular RNA.

5

13.   The method of claim 12, further comprising the step
of converting said RNA to cDNA.

10   14.   The method of claim 1, wherein said sample is
comprised of a body tissue or body fluid.

15.   The method of claim 14, wherein said body fluid,
15   comprising: peripheral blood, lymph fluid, ascites,
serous fluid, pleural effusion, sputum, cerebrospinal
fluid, lacrimal fluid, stool or urine.

20   16.   The method of claim 14, wherein said body tissue,
comprising bone marrow aspirate, bone marrow biopsy,
lymph node aspirate, lymph node biopsy, spleen tissue,
fine needle aspirate, skin biopsy or organ tissue biopsy.

25
.17.   The method of claim 2, wherein said detecting is by
gel electrophoresis.

30   18.   The method of claim 2, wherein said detecting is by
chromatography.

19.   The method of claim 17, further comprising the step
35   of Southern blotting.

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20.   The method of claim 1, wherein said sample is of human origin.

5   21.   A kit for use in detecting melanoma or breast cancer cell cells in a biological sample comprising:

         (a)   pairs of primers for amplifying nucleic acids
                corresponding to the genes for tyrosinase,
10   MAGE-3, MUC18, p97, MAGE-1, GalNAc and ß-HCG;
                and

         (b)   containers for each of said primers.

15

     22.   The kit of claim 21, further comprising enzymes and reagents for the preparation of cDNA's.

20   23.   The kit of claim 21, further comprising enzymes and reagents for radiochemical or chromophoric labeling of nucleic acids.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68   C12P19/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 00603 (THE TRUSTEES OF PRINCETON UNIVERSITY) 6 January 1994 see page 10, line 26 - page 11, line 8 ---	1-3, 10-20
X	AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol. 94, no. 4, October 1990, page 507 XP000576592 COX C ET AL: "tumor marker sensitivity single versus multiple markers in patients with breast carcinoma" see abstract 77 ---	1-3, 10-20
X	ISREAL JOURNAL OF MEDICAL SCIENCES, vol. 17, no. 9-10, September 1981, pages 865-8, XP000576568 SULITZEANU D: "markers in breast cancer" see the whole document ---	1-3, 10-23

-/--

|X| Further documents are listed in the continuation of box C. |X| Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 July 1996	29.07.96

| Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016 | Authorized officer

Osborne, H |

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IMMUNOLOGY SERIES, vol. 53, 1990, pages 39-54, XP000576588 MERCER D.: "use of multiple markers to enhance clinical utility" see page 44, paragraph 6 - page 46, paragraph 1 ---	1-3, 10-20
X	GYNECOLOGICAL AND OBSTETRIC INVESTIGATION, vol. 34, no. 2, 1992, pages 65-72, XP002008750 FARGHALY S.: "tumor markers in gynecologic cancers" abstract and page 71 ---	1-3, 10-20
A	THE LANCET, vol. 338, 16 November 1991, pages 1227-9, XP002008751 SMITH B. ET AL: "detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction" see the whole document ---	1,23
A	EP,A,0 520 794 (F. HOFFMANN LA-ROCHE AG) 30 December 1992 see the whole document ---	1,23
A	WO,A,90 09456 (BALAZS) 23 August 1990 see claims 1-13 -----	1-3

2

INTERNATIONAL SEARCH REPORT

.ormation on patent family members

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9400603	06-01-94	AU-B-	4544393	24-01-94
		AU-B-	4545193	24-01-94
		CA-A-	2137822	06-01-94
		EP-A-	0654092	24-05-95
		JP-T-	8500729	30-01-96
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